Facilitated Diffusion Properties of Melibiose Permease in *Escherichia coli* Membrane Vesicles

**RELEASE OF CO-SUBSTRATES IS RATE LIMITING FOR PERMEASE CYCLING**

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The mechanism of melibiose symport by the melibiose permease of *Escherichia coli* was studied by looking at the modifications of the facilitated diffusion properties of the permease which arise upon substitution of the coupled cations (H⁺, Na⁺, or Li⁺). Kinetic analysis of melibiose influx and efflux down a concentration gradient, exchange at equilibrium, and counterflow were examined in de-energized membrane vesicles resuspended in media allowing melibiose to be co-transported with either H⁺, Na⁺, or Li⁺. The data show that the maximal rates of melibiose efflux coupled to either H⁺, Na⁺, or Li⁺ are between 10 and 40 times faster than the corresponding influxes. This suggests that the permease functions asymmetrically. Cross-comparison between the rates of net [H]melibiose entry during the influx reactions coupled to either cation and corresponding unidirectional sugar influx down exchange and counterflow reactions leads to the conclusions that: 1) the step involving release of the co-substrates from the permease on the inner surface of the membrane is sequenced (sugar first and then coupled cation); 2) this step is rate determining for cycling of the permease. The Na⁺-melibiose passive flux data indicate in particular that release of Na⁺ ions rather than release of sugar into the intravesicular space is the slowest step during permease cycling. This property would hamper net passive Na⁺-melibiose influx but should allow exchange of sugar without concomitant exchange of the coupled cation. Finally, evidence is provided suggesting that the relative rates of release of the two co-substrates from the permease on the inner membrane surface varied considerably in relation to the identity of the coupled cation.

Accumulation of a wide variety of nutrients in bacteria is mediated by co-transport systems or symporters and results from obligatory coupling between uphill entry of solutes and favorable, downhill entry of cations. In many bacterial transport systems, protons are the obligatory coupling cations. A typical and extensively studied example of such a bacterial transport system is the lactose permease of *Escherichia coli* (1–3). In other microorganisms such as halophilic or marine strains, however, the transport reaction is exclusively coupled to Na⁺ instead of to H⁺ (reviewed in Ref. 4).

Between these two extremes, the melibiose permease which transports α-galactosides (and also some β-galactosides) in *E. coli* (5, 6) and *Salmonella typhimurium* (7) is of special interest because it can alternatively use H⁺, Na⁺, or Li⁺ as coupled cation depending on the sugar substrate and cationic environment. Thus, binding onto the carrier (8, 9) and accumulation of different disaccharides by the melibiose† permease (6, 10, 11) are stimulated by the presence of Na⁺ (or Li⁺) ions; conversely, these substrates enhance the uptake of Na⁺ and Li⁺ ions (5, 12). These observations are consistent with a carrier mechanism acting as a Na⁺- or Li⁺-melibiose symport. Other evidence shows that the melibiose permease can function as a H⁺-symport system. For example, it has been demonstrated that H⁺ enter concomitantly with the sugar during melibiose pulse experiments in de-energized cells resuspended in Na⁺-free solution (5). Importantly, genetic (13, 14) and functional evidences (5, 9, 15) suggest that one and the same permease catalyses the three different modes of symport activity.

It is worth mentioning that appreciable variations of the co-transport reaction occur in relation to the chemical nature of the coupled cation. Thus, although α- and some β-galactosides are co-transported with Na⁺, only sugars with an α configuration are sympported with H⁺ (16). Furthermore, the apparent dissociation constant for α-galactosides binding on the permease (8, 9) or the apparent affinity constant for transport (6, 11) are significantly higher in the presence of H⁺ than in that of Na⁺. Also, the maximal rate of Na⁺-melibiose co-transport in cells is about three times faster than the Li⁺-coupled reaction (6). Finally, the carrier-mediated influx of melibiose down a concentration gradient in de-energized membrane vesicles is one order of magnitude slower in Na⁺ than in H⁺ (11). Such a cation dependence, if studied more systematically, would certainly provide insight into the role of the coupled cation in the mechanism of symport. This would be particularly true for the carrier-mediated flux properties (influx, efflux, exchange at equilibrium, and counterflow) and could be expected, as in the case of the lactose permease (17–20), to provide mechanistic insight into individual steps of the sugar-cation co-transport reaction. Moreover, knowledge of the characteristics of these passive movements was recently shown to be extremely useful in establishing the functional importance of given amino acid residues of the

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1. The abbreviations used are: melibiose, 6-O-a-D-galactopyranosyl-D-glucose; TMG, β-thiogalactoside; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; 2,4-DNP, 2,4-dinitrophenol; NEM, N-ethylmaleimide, ΔH*, electrochemical proton gradient; ΔH; ΔNa, and ΔLi*, chemical gradient of H*, Na⁺ and Li⁺; ΔV, electrical potential.
lactose permease engineered by site-specific mutagenesis (1, 2).

The aim of the present paper was therefore to analyze how permutation of the three symported cations (H\(^+\), Na\(^+\), and Li\(^+\)) affects the facilitated diffusion properties (influx, efflux, and exchange at equilibrium and counterflow activity) of the melibiose permease in de-energized membrane vesicles from *E. coli*. The results reported below show that the nature of the coupled cation strongly influences the ability of the melibiose permease to promote carrier-mediated influx, exchange, and counterflow activities. They also suggest that the permease is functionally asymmetrical. Finally, comparative analysis of the Na\(^+\)/sugar fluxes catalyzed by the permease suggests that cycling of the permease is rate limited by the step corresponding to release of the coupled cation from the carrier on the inner surface.

**EXPERIMENTAL PROCEDURES**

**Growth of Cells and Preparation of Membrane Vesicles—** *E. coli* RA11 strain has a temperature stable, inducible melibiose transport but lacks α-galactosidase activity and has a deletion for the Lac Y gene (10). Cells were grown in minimal medium in the presence of 10 mM melibiose as already described (10). Right-side-out membrane vesicles were prepared essentially as described by Kaback (21), resuspended in 0.1 M potassium phosphate (pH 6.6), and stored in liquid nitrogen for subsequent use.

**Transport Assays in Membrane Vesicles—** Thawed membrane vesicles were washed and equilibrated in 0.1 M potassium phosphate buffer, 10 mM MgSO\(_4\) buffered solution (pH 6.6) prepared with high purity salts (Merck) to reduce Na\(^+\) contamination (20 mM Na\(^+\)). This medium, subsequently referred to as H\(^+\) medium, was used to measure H\(^+\)-coupled influx, efflux, and exchange of melibiose. For determination of Na\(^-\)+ or Li\(^-\)+-coupled movements of melibiose, NaCl, or LiCl were added to the H\(^+\) medium to a final concentration of 10 mM; these media are referred to as Na\(^+\) or Li\(^+\) media.

In the case of carrier-mediated influx experiments, membrane vesicles were resuspended at about 5 mg/ml in the corresponding medium and incubated in the presence of FCCP (10 \(\mu\)M) and Monensin (0.75 \(\mu\)M) for 30 min at room temperature. Then, 50-\(\mu\)l aliquots were incubated at 25 °C and the uptake reaction initiated by adding \([\text{H}]\text{melibiose (40 and 2.5 mCi/mmol) at concentrations ranging from 0.05 to 2.4 mM in Na\(^+\) or Li\(^+\) medium and up to 20 mM in H\(^+\) medium. At various intervals during the first 15 s, the samples were diluted with 2 ml of saline solution of equilibration, filtered on GF/F glass fiber filters (Whatman) and the filters washed once with 4 ml of the same saline solution. For melibiose efflux or exchange experiments, washed membrane vesicles were concentrated to about 30 mg of membrane protein/ml in their respective media in the presence of FCCP and Monensin. Aliquots of concentrated \([\text{H}]\text{melibiose (2-5 mCi/mmol)}\) were added to give the desired sugar concentrations, and the suspensions were left to equilibrate at room temperature for 40 min and then overnight at 4 °C. Efflux and exchange reactions were initiated by diluting 2.5-\(\mu\)l aliquots of sugar-loaded vesicles in 2 ml of corresponding H\(^+\), Na\(^+\), or Li\(^+\) medium devoid of sugar (for efflux measurements) or supplemented with an equimolar concentration of unlabeled sugar for exchange measurements. At given times after dilution, samples were filtered, and the filters were washed and assayed for radioactivity. Countercurrent experiments were performed as described by Kaczorowski et al. (17) by diluting concentrated membrane vesicles loaded with unlabeled sugar in media containing given concentrations of \([\text{H}]\text{melibiose (10 - 20 mCi/mmol)}\). The countercurrent reaction was interrupted at given times after dilution by filtering the samples as described for influx. For measurements of Na\(^-\)+ movements coupled to melibiose efflux, membrane vesicles were previously loaded with either Na\(^+\) (10 mM, 3 mCi/mmol) and unlabeled sugar or unlabeled Na\(^+\) and \([\text{H}]\text{melibiose (30 mM, 5 mCi/mmol)}\) according to the loading protocol described above (except that Monensin was omitted). After dilution of concentrated samples (2.5 \(\mu\)l) in media devoid of both Na\(^+\) and sugar, rates of efflux of intravesicular Na\(^+\) or \([\text{H}]\text{melibiose were monitored using a filtration assay.}

Rates of influx, efflux, and exchange were corrected for passive permeability components by performing parallel experiments on membrane vesicles previously incubated with 1 mM N-ethylmaleimide for 30 min at room temperature.

**Efflux and Exchange in Intact Cells—** Experiments were performed on cells treated with EDTA in order to improve the uncoupling efficiency of protonophores (2-4 DNP and FCCP) and monensin. EDTA treatment was carried out at 37 °C for 2 min as described by Padan et al. (28). Cells were then thoroughly washed by filtration as described and incubated in the presence of 2-4 DNP (1 mM), FCCP (10 \(\mu\)M), and monensin (0.75 \(\mu\)M) for 30 min at room temperature, concentrated in the same solution to about 30 mg of cell protein/ml, and divided into three batches. The one to be used for determinations of H\(^+\)-coupled melibiose fluxes was supplemented with \([\text{H}]\text{melibiose (40 mM, 2.5 mCi/mmol), and the others to be used for determinations of Na\(^-\)+ or Li\(^-\)+-coupled melibiose fluxes were supplemented with 40 mM of labeled sugar and 10 mM of NaCl or LiCl. Efflux and exchange reactions in cells were performed in the presence of 2-4 DNP and measured as described for membrane vesicles.

**Proteins—** Proteins from cells solubilized in the presence of 0.2% sodium dodecyl sulfate or from membrane vesicles were estimated by the technique of Lowry et al. (22) using serum albumin as a standard.

**Materials—** \([\text{H}]\text{melibiose (2.7 Ci/mmol)}\) was titrated by catalytic exchange in the Service des Molecules Marques (Commissariat Energetique Atomique, France); \(\text{NaCl (carrier-free) was from C.E.A. (France). Stock solutions of FCCP (Boehringer), and Monensin (Sigma) were prepared in MeSO. N-Ethylmaleimide (Behring Diagnostics) and 2-4 DNP (Prolabo) solutions were prepared shortly before use.**

**RESULTS**

Kinetic analysis of the carrier-mediated melibiose influx, efflux, and exchange were made on de-energized membrane vesicles resuspended in 100 mM potassium phosphate buffer (pH 6.6) containing either less than 20 mM Na\(^+\), 10 mM Na\(^+\), or 10 mM Li\(^+\) ions (referred to below as H\(^+\), Na\(^+\), and Li\(^+\) media). Previous transport and binding studies (6, 9) have shown that in these conditions the melibiose transport functions as either a H\(^+\)-, a Na\(^-\)+-, or a Li\(^-\)+-melibiose symport. In our experiments, possible self-perturbation of melibiose influxes or effluxes by transient Δψ or ΔpH (ΔpNa or ΔpLi) induced by the net movements of co-transported cationic species was prevented by pretreating the vesicles with FCCP (10 \(\mu\)M) and Monensin (0.75 \(\mu\)M).

**Carrier-mediated Melibiose Influx—** Table I shows that the melibiose permease catalyzes a significant passive H\(^+\)-melibiose influx. The maximal rate of this passive H\(^+\)-melibiose

**Table I**

<table>
<thead>
<tr>
<th>K (_r)</th>
<th>V (_{\text{max}})</th>
<th>K (_d)</th>
<th>V (_{\text{max}})</th>
<th>K (_r)</th>
<th>V (_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Melibiose H(^+)</em></td>
<td><em>Melibiose Na(^+)</em></td>
<td><em>Melibiose Li(^+)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influx</td>
<td>10</td>
<td>80</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>Efflux</td>
<td>40</td>
<td>400</td>
<td>12</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>Exchange</td>
<td>20</td>
<td>80</td>
<td>12</td>
<td>60</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
influx was comparable to the influx measured in the presence of an energy source (11). By contrast, no, or very limited, passive Na⁺ (or Li⁺)-melibiose influx was detected in de-energized conditions. Identical results were observed in membrane vesicles containing or without 10 mM internal Na⁺ ions. These influx values are considerably lower than the rates of Na⁺ or Li⁺ transport of melibiose in energized conditions ($V_{\text{max}}$ are 25 and 7 nmol/mg of protein.min, respectively). A similar reduced rate of passive Na⁺-TMG influx by the melibiose permease has already been reported (8). Table I also indicates that the apparent affinity constant ($K_a$) for passive H⁺-melibiose influx is about 50 times higher than the $K_a$ for passive Na⁺ or Li⁺-melibiose influx. The difference in the $K_a$ values between the H⁺-melibiose influx and the Na⁺ or Li⁺-melibiose influxes might reflect, at least in part, the 20-fold lower affinity of the permease for the sugar when the coupled cation is H⁺ than when it is Na⁺ or Li⁺ (9).

Carrier-mediated Melibiose Efflux and Exchange—Previous work on different bacterial permeases (8, 17, 23) has shown that passive, carrier-mediated efflux and exchange reactions can be studied by measuring the rate of intravesicular disappearance of labeled sugar after dilution of sugar-preloaded membrane vesicles in media lacking sugar (efflux) or media containing an equimolar concentration of unlabeled sugar (exchange at equilibrium). This protocol was used to analyze the effects of substitution of the symported cation on the efflux and exchange properties of the melibiose permease. In the typical experiment illustrated in Fig. 1, membrane vesicles pre-equilibrated in either H⁺, Na⁺, or Li⁺ medium and loaded with 40 mM [³²H]melibiose were diluted in the corresponding saline medium unsupplemented with melibiose (EF, closed symbols) or containing 40 mM melibiose (EX, open symbols).

The data presented in this figure show that all individual rates of melibiose efflux and exchange can be satisfactorily fitted by first order relationships. Also, and irrespective of the coupling cation, pretreatment of membrane vesicles with 1 mM NEM (or 50 mM N-naphthylmaleimide, not shown) led to a 85–95% reduction of the rates of both melibiose efflux and exchange (filled squares). Since the SH reagent completely inhibited the melibiose permease activity (9, 10, 24), it follows that most of the outward movement of labeled sugar observed in our experiments was carrier-mediated. In addition to these preliminary remarks, three major conclusions can be drawn from these experiments. First, examination of the left panel of Fig. 1 shows that in H⁺ medium, the rate of H⁺-coupled melibiose efflux is at least twice that of the exchange rate ($V_E$ and $V_X$ are 131 and 59 nmol/mg of protein.min, respectively). The situation is thus the reverse of that reported for the lactose symport in which it was shown that lactose efflux is much slower than lactose exchange (17, 20). Second, it is apparent from the middle panel that the rates of Na⁺-melibiose efflux and exchange are essentially similar (46 nmol/mg of protein.min). This finding resembles Cohn and Kaback's (8) observation that Na⁺-TMG efflux and exchange catalyzed by the melibiose permease are nearly identical. Finally, the right panel in Fig. 1 indicates that melibiose efflux is significant in the presence of Li⁺ ions, and its rate (48 nmol/mg of protein.min) is similar to that measured in the presence of Na⁺ ions. Unexpectedly, however, no, or a negligible melibiose exchange takes place in the Li⁺ medium. These data clearly demonstrate that, at that given internal sugar concentration, the relative rates of melibiose efflux and exchange are considerably influenced by the identity of the coupled cation.

Fig. 2 shows the results of the kinetic analysis of melibiose efflux and exchange in the presence of each coupling cation and as a function of the sugar concentration. The kinetic parameters of the different efflux and exchange reactions are given in Table I. The data first indicate that at all melibiose concentrations tested, H⁺-melibiose efflux remains much greater than H⁺-melibiose exchange, Na⁺-melibiose efflux and exchange are of similar magnitude, and finally Li⁺-melibiose exchange is negligible beside Li⁺-melibiose efflux. Because these conclusions still hold in the presence of saturating concentration of sugar (Table I), one can conclude that the controlling factors of the melibiose exchange are the sugar concentration and the identity of the coupled cation.

FIG. 1. Influence of the nature of the symported cation (H⁺, Na⁺, or Li⁺) on the relative rates of melibiose efflux and exchange by the melibiose permease. E. coli RA11 membrane vesicles were equilibrated and concentrated to ~30 mg/ml in either H⁺, Na⁺ (10 mM), or Li⁺ (10 mM) medium (pH 6.6) containing FCCP (10 mM) and monensin (0.75 μM). A small aliquot of [³²H]melibiose (5 μCi/ml) was added to each suspension to a final concentration of 40 mM, and intravesicular equilibration was achieved by overnight incubation at 4 °C. At the onset of the experiments illustrated in each panel, 2.5-μl aliquots of loaded membrane vesicles were diluted 800-fold in the corresponding H⁺, Na⁺, or Li⁺ medium either supplemented with 40 mM of unlabeled sugar (exchange = EX, ○) or devoid of melibiose (efflux = EF, ◦). At given times after dilution, the samples were filtered and the remaining intravesicular radioactivity measured. And dotted line correspond to efflux (or exchange) experiments performed in vesicles pretreated with 1 mM NEM. Data corrected for nonspecific contributions are presented as percent of initial radioactivity measured in samples which have been filtered immediately after dilution (internal melibiose concentration of 40 ± 4 mM). All measurements were performed on the same membrane vesicle preparation.

FIG. 2. Kinetic analysis of H⁺, Na⁺, and Li⁺-melibiose efflux and exchange in de-energized membrane vesicles. Membrane vesicles were pre-equilibrated in either H⁺, Na⁺, or Li⁺ medium (pH 6.6), concentrated, and loaded with given concentrations of [³²H] melibiose (from 5 to 20 μCi/mmol according to the concentration) as described in Fig. 1. FCCP (10 μM) and monensin (0.75 μM) were present throughout the experiments. Efflux (○) and exchange (□) rate measurements were performed as described in Fig. 1 except that during exchange the concentration of unlabeled melibiose in the diluting medium equals that of the loading solution. Efflux and exchange rates were corrected for the contribution of NEM-insensitive components.
cation-dependent variability in pattern of the relative rates of sugar efflux and exchange reflects a variation of some step(s) associated with translocation of the loaded (or unloading carrier) or release of the co-substrates from the carrier into the internal space. In addition to this conclusion, Fig. 2 and Table I indicate that, at infinite substrate concentration, the relative rates of melibiose efflux are in order $H^+$-melibiose efflux $>$ $Na^+$-melibiose efflux $\geq$ $Li^+$-melibiose efflux. The opposite sequence is found for the $K_t$ values (Table I). Thus, substitution of $H^+$ for $Na^+$ or $Li^+$ leads to a significant modification of the properties of the melibiose permease, i.e. a shifting of the carrier from a low affinity-high capacity efflux system to a relatively high affinity-low capacity efflux system. Lastly, comparison of the kinetic parameters of the $H^+$, $Na^+$, and $Li^+$ efflux rates with the corresponding influxes in Table I indicates that the melibiose permease functions asymmetrically. Indeed, in the presence of any coupled cations, the maximal rate of melibiose efflux is more than 10 times higher than the $V_{\text{max}}$ for the corresponding influx. Conversely, the $K_t$ value for the efflux is from 3 to 50 times higher than the corresponding $K_t$ for the influx. It is important to note, however, that the estimated $V_{\text{max}}$ and $K_t$ values are such that the Haldane relationship ($V_{\text{max}}/K_t$ is virtually constant for influx and efflux of $H^+$, $Na^+$, and $Li^+$-melibiose). This is to be expected from a permease which catalyzes equilibration of the sugar in de-energized conditions.

Three further observations merit mentioning briefly. First, absolute $V_{\text{max}}$ values for melibiose efflux and exchange in $H^+$, $Na^+$, or $Li^+$ medium varied considerably from one membrane preparation to the other. In all cases, however, the pattern of relative rates of efflux and exchange illustrated in Fig. 1 was seen consistently. Second, the concentration of $Na^+$ and $Li^+$ of 10 mM chosen during determination of the $Na^+$- or $Li^+$-coupled melibiose fluxes described above is saturating. Indeed, increasing the $Na^+$ concentration up to 50 mM did not appreciably modify the influx, efflux, and exchange rates when compared with those recorded at 10 mM $Na^+$ (not shown). Finally, the $H^+$-, $Na^+$- or $Li^+$-coupled efflux and exchange of melibiose taking place in intact cells loaded with 40 mM of [$^3H$]melibiose exhibited exactly the same dependence on the coupled cation as those described in membrane vesicles (not shown).

**Melibiose Counterflow in the Presence of $H^+$, $Na^+$, and $Li^+$**—The observation that $Na^+$-melibiose influx is negligible whereas $Na^+$-melibiose exchange is considerable is puzzling since both reactions involve an inward movement of sugar. This unexpected finding was further investigated by comparing the rate of sugar entry during an entrance counterflow experiment and net entry of $Na^+$/sugar (influx) at a similar external concentration of labeled sugar. To this end, membrane vesicles loaded, or not, with 20 mM unlabeled melibiose and 10 mM $Na^+$ were diluted in a medium containing 10 mM $Na^+$ and 0.4 mM melibiose (i.e. a concentration twice as high as the $K_t$ of active $Na^+$ melibiose transport rate) and the sugar entry measured as a function of time. Fig. 3 shows that the unidirectional inward flux of sugar during counterflow is more than 20 times faster than the rate of net $Na^+$ melibiose influx. One can conclude that the particular step limiting the inward movement of sugar during net melibiose influx is not involved in exchange or counterflow reactions. Similar comparison between net melibiose influx and counterflow were performed in the presence of $H^+$ and $Li^+$ as the coupled cations. The initial rates of these reactions are illustrated in the inset of Fig. 3. For $H^+$-coupled reactions, the initial rate of sugar entry during counterflow is higher than during influx; this difference, however, disappears at higher external concentrations of sugar (not shown). By opposition to $Na^+$-coupled reactions it seems that net $H^+$-melibiose influx exchange and counterflow are limited by a common step. Finally, the inset shows that the permease does not exhibit $Li^+$-melibiose counterflow activity. Compared with the $Na^+$-melibiose counterflow activity, these latter data suggest that the coupled cation has effects on the ternary complex other than simply changing the net charge of the carrier.

$Na^+$ Sugar Stoichiometry of the Melibiose Carrier—Previous analysis of the $Na^+$/sugar stoichiometry of the bacterial melibiose permease (7, 27), have shown that determination of the coupling ratio between $Na^+$ and sugar influxes in energized conditions is complicated by the extremely fast recirculation of $Na^+$ ions via the $\Delta\gammaH^+$ activated $Na^+/H^+$ antiporter (25). The observations reported above which show that the melibiose permease catalysts a significant sugar efflux in de-energized vesicles resuspended in $Na^+$ medium make it possible to analyze the $Na^+$/sugar-coupling ratio in the absence of considerable $Na^+$ recycling. Concomitant unidirectional $Na^+$ and melibiose movements were therefore compared in conditions of efflux using membrane vesicles loaded with either 10 mM $^{22}Na^+$ and 30 mM unlabeled melibiose or 10 mM $Na^+$ and 30 mM [$^3H$]melibiose (Fig. 4). In these experiments, $Na^+$-free solutions were used as diluting media in order to avoid time-dependent variations of the internal specific activity of $^{22}Na^+$ consecutive to inward movements of unlabeled $Na^+$ ions. Fig. 4A shows that the rate of $^{22}Na^+$ efflux from melibiose-loaded membrane vesicles (closed triangles) is much higher than that of $^{22}Na^+$ efflux from vesicles not loaded with melibiose (open triangles). Two independent observations indicate that the increase in rate of $^{22}Na^+$ efflux is mediated by the melibiose carrier. First, no such acceleration is recorded in vesicles pretreated with 1 mM NEM, a reagent which inhibits the melibiose porter (24) but not the $Na^+$ pathways, including the $Na^+/H^+$ antiporter (26) in membrane vesicles. Second, lactose, which is a poor substrate for the melibiose permease fails to promote acceleration of $^{22}Na^+$ efflux. It is
the characteristics of some partial reactions of the lactose H+-lactose symport mechanism, first in membrane vesicles when Li' is the coupling cation. It is important to note that their dependence on the coupled cation can be used in the experimental protocol described in Fig. 1, membrane vesicles pretreated with NEM; Panel B: 10 mM NaCl, 30 mM lactose; ▲, 10 mM NaCl, no sugar added. Panel C: 10 mM NaCl, 30 mM [H]melibiose (5 mgCi/mmol); ■, same, but NEM-treated membranes. 2.5-μl aliquots of membrane vesicles loaded either with labeled Na' or melibiose were diluted in 2 ml medium devoid of Na' and sugar. The rates of internal [22Na'] or [H]melibiose decrease after dilution were corrected for nonspecific binding on the filters and expressed as percent of radioactivities present at zero time (see Fig. 1).

thus evident that the NEM-sensitive fraction of the [22Na'] efflux occurring in melibiose-loaded vesicles is mediated by the melibiose porter. On this basis, comparison of the NEM-sensitive fraction of [22Na'] efflux (72 nmol/mg-protein-min) with the NEM-sensitive fraction of [H]melibiose efflux calculated from the data in Fig. 4B (90 nmol/mg protein-min) gives a Na'/sugar-coupling ratio of 0.88. Coupling ratio values ranging from 0.8 to 1.3 were found in different experiments in which the intravesicular melibiose concentration varied between 10 and 30 mM. The Na'/sugar stoichiometry of the melibiose efflux reaction is thus close to unity.

DISCUSSION

The experiments reported in this study demonstrate essentially that substitution of Na' for H' or Li' as a coupling cation in the mechanism of symport of melibiose by the melibiose permease results in considerable modification of the facilitated diffusion properties (influx, efflux, and exchange) and counterflow activity of the permease. They also revealed that the permease exhibits asymmetrical functioning with each of the coupled cations.

It is indeed clear from Figs. 1–3 and Table I that, for example, downhill sugar entry into de-energized membrane vesicles is about 20 times faster when the co-transported cation is H+ than when it is Na+ or Li+. Also, whereas the carrier displays melibiose exchange and counterflow activities in the presence of Na+, none of these transport reactions is catalyzed when Li+ is the coupling cation. It is important to stress that similar cationic dependence of the melibiose efflux and exchange properties are observed in intact cells.

The passive melibiose flux properties and counterflow activity and their dependence on the coupled cation can be used to probe the melibiose symport reaction. Previous studies of the H+-lactose symport mechanism, first in membrane vesicles by Kaczorowski et al. (17, 18) and then in cells (20), have indeed shown that analysis of the facilitated diffusion properties in de-energized systems provides important insight into the characteristics of some partial reactions of the lactose transport mechanism. The sequence of events involved in the various passive transport reactions catalyzed by a given symport system, i.e. co-substrate binding on (or release from) the carrier and also reorientation steps of the loaded and unloaded forms of the carrier, is schematically represented in Fig. 5. In this kinetic scheme, the choice of a 1:1 cation/sugar stoichiometry is entirely justified when Na' is the coupled cation in view of the Na'/sugar-coupling ratio measured either during downhill efflux (Fig. 4), or during influx (6) or from the sugar-binding studies on the melibiose permease (9). Since the binding studies also suggested first that Li+ and Na+ have equivalent activating effects on sugar-binding activity and second that H+ competes with Na' (and Li') for the same cationic site on the permease, it has been assumed in the following that the H+ and Li+/sugar stoichiometries are also 1:1.

One of the most striking properties of the melibiose permease is the asymmetrical functioning of the permease. Indeed, comparison of the kinetics of downhill melibiose influx and efflux with H+, Na+, or Li+ as coupling cation indicates that effluxes are between 10 and 40 times faster than corresponding influxes (Fig. 2 and Table I). Since efflux still exceeds influx at saturating concentrations of co-substrates, reduced rates of influx result either from a slow rate of inward translocation of the ternary complexes (step 2), or from a reduced rate of co-substrate release from the carrier on the inner surface (step 3), or finally from slow rates of reorientation of the unloaded carrier (step 4). Although there are good indications that return of the unloaded carrier is rate limiting in the case of lactose permease (18, 20), cross-comparison of the Vmax of H+, Na+, and Li+-melibiose influxes (Table I) excludes this possibility in the case of melibiose permease. Thus, return of the unloaded carrier is a partial step common to the relatively fast H+-melibiose influx reaction and to the very slow Na' (or Li')-melibiose influx one. Furthermore, the rate of return of the unloaded carrier is independent of the coupled cation and is at least as fast as the rate of the overall reaction of H+-melibiose influx. It follows that return of the unloaded carrier is about 20 times faster than the overall rate of sugar influx coupled to either Na' or Li'. It cannot therefore be rate limiting during the slow Na' or Li'-melibiose influx cycle. This conclusion agrees with that drawn earlier by Cohn and Kaback (8) from comparison of passive Na'.TMG efflux and exchange properties catalyzed by melibiose permease. Comparison of the rate of H+-melibiose influx (steps 1–4) and exchange (steps 1–3 and 1', 2', 3') at saturating concentrations of substrate also suggests that return of the unloaded carrier cannot be the slowest step during H+-melibiose influx.

![Fig. 5. Panel A: schematic representation of partial steps involved in melibiose carrier cycle: step 1, binding (or release, 1') of co-substrates to the carrier on the outer surface of the membrane; step 2, inward (or outward, 2') translocation of the ternary complex; step 3, release (or binding, 3') of the co-substrates from the carrier on the inner surface; and finally step 4, outward (or inward, 4') translocation of the unloaded carrier. C, S, and X' correspond to the carrier protein, melibiose, and coupled cations (H', Na', or Li'), respectively. Panel B: sequence of release of the two co-substrates from the melibiose carrier on the outer surface of the membrane.](image-url)
Thus, were the return of the unloaded carrier (step 4) rate limiting for the \( H^+ \)-melibiose influx, exchange would be faster than influx since this step is not involved in the exchange reaction and steps 1', 2', and 3' which are involved in this exchange are fast (see efflux data Table I). In fact, exchange and influx rates are of comparable magnitude (Table I). One can thus conclude that the rate-limiting factor for \( H^+ \)-melibiose influx, as for \( Na^+ \)- or \( Li^+ \)-melibiose influx, is either step 2 or 3, i.e. the inward movement of the ternary complex or the release of co-substrates in the intravesicular space.

Comparison of the \( Na^+ \)-melibiose influx, exchange, and counterflow rates at saturating concentrations of substrate (Table I, Fig. 3) eliminates the possibility that inward translocation of the ternary complex (step 2) is limiting for sugar entry during the \( Na^+ \) influx reaction. Thus, whereas there is significant unidirectional sugar entry during \( Na^+ \)-melibiose counterflow (Fig. 3), there is no, or negligible, net inward movement of sugar coupled to \( Na^+ \) ions during sugar influx down a concentration gradient. The high rate of \( Na^+ \)-melibiose exchange also implies that the unidirectional sugar entry component is large during this exchange reaction. These findings indicate that the inward translocation of the loaded carrier is not slow enough to account for the very reduced rate of net melibiose entry during influx down a concentration gradient. We therefore conclude that the rate of release of co-substrates from the carrier on the inner surface (step 3) must control the overall \( Na^+ \)-melibiose influx reaction rate.

Further insight into this control mechanism is obtained by considering the sequence of co-substrate defixation on the inner surface shown in Fig. 5B. It is indeed possible to account for the large difference in the rate of the various \( Na^+ \)-coupled reactions if it is assumed (i) that release of co-substrates from the carrier on the inner surface (step 3) is an ordered process (sugar released first, \( Na^+ \) last) and (ii) that release of \( Na^+ \) ions from the carrier on the inner surface occurs at a very slow rate. Such a model predicts that the reduced rate of \( Na^+ \) release into the intravesicular space would indeed limit the overall rate of counterclockwise cycling of the carrier during influx. On the other hand, the proposal that melibiose is released first and at a greater rate than that of the coupled cation opens the possibility of a sugar-sugar exchange reaction (or counterflow) and thus catalysis of an unidirectional sugar movement during these reactions. Sugar-sugar exchange would however take place without concomitant exchange of a coupled cation. Direct evidence in favor of this interpretation has been obtained by measuring isotopic \( Na^+ \) movements during the exchange reaction and will be presented in a forthcoming publication. Indications that sugar-sugar exchange could be dissociated from coupled cation exchange were also found in the case of lactose permease (7).

Interpretation of the \( H^+ \)- and \( Li^+ \)-melibiose flux data in terms of the kinetic scheme derived from the \( Na^+ \)-melibiose flux data appears possible by introducing the assumption that the characteristics of release of the two co-substrates vary according to the coupled cation. Thus, the quite significant rate of \( H^+ \)-melibiose influx implies that defixation of \( H^+ \) from the carrier on the inner surface during this reaction is more frequent than release of \( Na^+ \) or \( Li^+ \) during \( Na^+ \)- or \( Li^+ \)-coupled melibiose influx. Furthermore, the observation that \( H^+ \)-melibiose exchange has a rate comparable with \( H^+ \)-melibiose influx suggests that release of \( H^+ \) is even faster than release of the sugar in the internal space. Concerning the \( Li^+ \)-coupled reactions, the absence of either influx, exchange, or counterflow activities indicates that release of the sugar does not occur during these particular reactions in the presence of \( Li^+ \). An attractive explanation would be to suppose that the strength of interaction between coupled cation and carrier conditions the rate of release of both co-substrates. Some evidence in favor of this working hypothesis will also be presented in a forthcoming paper.

In conclusion, the data presented in the present study strongly suggest that release of the co-transported species from the melibiose carrier into the internal space determines the rate of cycling of the permease in the absence of an electrochemical gradient across the membrane.

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